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(54) Title: PREVENTION OF PROTEIN AGGREGATION

(57) Abstract

A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and which are non-inhibitory to the biological activity of the target molecule when bound. The method includes the steps of mixing a denatured target molecule with a presumptive anti-aggregation molecule and then determining if the target molecules are prevented from self- or induced-aggregation. The nonaggregated target molecule coupled to the anti-aggregation molecule is then tested for bioactivity.

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PREVENTION OF PROTEIN AGGREGATION

TECHNICAL FIELD

5   The present invention relates to the use of
monoclonal antibodies, genetically engineered antibody
fragments and small peptides which mimic antigen
binding sites on the antibody for the prevention of
protein aggregation *in vivo* and *in vitro*.

10

BACKGROUND OF THE INVENTION

When proteins are synthesized they generally
must fold and assemble into a three dimensional form
15   to be active. Initially, it was thought that proper
folding was inherent in the amino acid sequence.
Recent work has shown that additional proteins, now
referred to as molecular chaperones, are required to
mediate the folding process or unregulated aggregation
20   of the polypeptides will occur preventing the
formation of functional proteins (Goloubinoff et al.,
1989; Welch, 1993). However, despite the existence of
chaperones, aggregation of protein still occurs *in
vivo* and can contribute to, or cause, various disease
25   states.
Other factors must contribute to the
occurrence of aggregation. These factors can include
mutations of naturally occurring chaperones inhibiting
function or allowing function with low efficiency
30   (Wetzel, 1994). Further, "pathological" chaperones
have been found which have been defined as "a group of
unrelated proteins that induce beta-pleated
conformation in amyloidogenic polypeptides"
(Wisniewski and Frangione, 1992). It would be useful
35   to be able to replace or augment the activity of the
chaperones where necessary and to counteract the

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activity of pathological chaperones when present.
Additionally, prion diseases have been shown to be
caused by, and/or associated with, a conformational
protein change wherein a protein normally having an
5   alpha helix conformation is converted to beta strands
which are collected into beta sheets (Prusiner, 1995).

Protein aggregation is of major importance
in biotechnology for the *in vitro* production of
recombinant proteins. *In vitro* aggregation limits the
10   protein stability, solubility and yields in production
of recombinant proteins. In cells during production
of recombinant proteins, aggregation is a major
impediment of recombinant proteins leading to
formation of inclusion bodies in the host cells
15   (DeYoung et al, 1993; Wetzel, 1994; Vandenbroeck et
al., 1993).

Further, *in vivo* protein aggregation or
precipitation is the cause, or an associated
pathological symptom, in amyloid diseases such as
20   Down's syndrome, Alzheimer's disease, diabetes and/or
cataracts, and in other disorders (DeYoung et al.,
1993; Haass and Selkoe, 1993; Wetzel, 1994; Prusiner,
1995).

Several peptides including β-amyloid, have
25   been shown to spontaneously self-associate, or
aggregate, into linear, unbranched fibrils in serum or
in isotonic saline (Banks and Kastin, 1992; Haass and
Selkoe, 1993). At least fifteen different
polypeptides are known to be capable of causing *in*
30   *vivo* different forms of amyloidosis via their
deposition in particular organs or tissues as
insoluble protein fibrils. Iron, zinc, chromium or
aluminum can participate in this aggregation (Bush et
al., 1994).

35   Molecular chaperones were initially
recognized as stress proteins produced in cells

requiring repair. In particular, studies of heat
shock on enzymes led the way to the discovery of
molecular chaperones that function not only during
cellular stress but normally to produce properly
5   folded proteins. The heat shock model is still one of
the models of choice in studying molecular chaperones
(Welch, 1993; Goloubinoff et al., 1989).

Molecular chaperones are a ubiquitous family
of proteins that mediate the post-translational
10   folding and assembly of other unrelated proteins into
oligomeric structures. They are further defined as
molecules whose functions are to prevent the formation
of incorrect structures and to disrupt any that form.
The chaperones non-covalently bind to the interactive
15   surface of the protein. This binding is reversed
under circumstances that favor the formation of the
correct structure by folding. Chaperones have not
been shown to be specific for only one protein but
rather act on families of proteins which have the same
20   stoichiometric requirements, i.e specific domains are
recognized by chaperones. This does not provide the
specificity required for therapeutic activity.

Further uses and descriptions of molecular
chaperones are set forth in PCT published
25   international patent applications 93/11248, 93/13200,
94/08012 and 94/11513 incorporated herein by reference
and in particular 94/08012 page 2 line 20 through page
5, line 14.

PCT published international patent
30   application 93/11248 discloses the use of a chaperone
in cell culture to promote efficient production of
protein in transformed cells by co-expression of the
chaperone molecule. This disclosure does not provide
specificity as to which proteins are protected except
35   through co-expression with the wanted protein nor does

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it provide information on how to use chaperones
therapeutically.

      PCT published international patent
application 93/13200 discloses the use of a chaperone
5 in a purification step for a recombinant protein
isolated from a cell culture and also a fusion protein
of the chaperone and recombinant protein. This
disclosure also does not provide specificity as to
which proteins are protected except through co-
10 expression with the wanted protein nor does it provide
information on how to use chaperones therapeutically.

      PCT published international patent
application 94/08012 discloses the use of a chaperone
in cell culture to promote increased secretion of an
15 overexpressed gene product in a host cell. This
disclosure does not provide specificity as to which
proteins are protected except through co-expression
with the wanted protein nor does it provide
information on how to use chaperones therapeutically.
20   PCT published international patent
application 94/11513 discloses the use of a vector
containing a molecular chaperone for treating
neoplasms. This disclosure does not provide
specificity as to which proteins are protected except
25 through co-expression with the wanted protein nor does
it provide information on how to use chaperones
therapeutically to treat diseases or syndromes which
involve protein aggregation.

      In each of the aforementioned publications,
30 the chaperones did not bind to native proteins and did
not redissolve aggregated proteins.

      Recent reports suggest that monoclonal
antibodies (mAb) can have chaperone-like activity.
The feasibility of using monoclonal antibodies to
35 assist in the *in vitro* refolding process of guanidine-
denatured S-protein was reported recently (Carlson and

Yarmush, 1992). Previously, Blond and Goldberg (1987)
used mAbs as a tool in the identification and
characterization of folding steps that involve the
appearance of local native-like structures in B_2

5   subunit of tryptophan-synthase. Since the mAb is
epitope specific, the use of a mAb provides more
specificity than molecular chaperones. Monoclonal
antibodies can be sought and engineered (Haber, 1992)
that bind to the particular epitope in the protein of

10 interest that is involved in the folding process.
The main difference between mAbs and
molecular chaperones is that the latter does not bind
to native proteins and is capable of interacting with
many different polypeptide chains without exhibiting

15 an apparent sequence preference (Goloubinof et al.,
1989). Moreover, chaperones suppress aggregation but
do not redissolve aggregate proteins already present.
Similar behavior was recently reported for α-crystalin
which, similar to other chaperones, does not react

20 with active proteins, but forms a stable complex with
denaturing or partially unfolded proteins, stabilizing
against further aggregation (Rao et al., 1994).
Aggregated amyloid β-protein (βA4) is a
major constituent of the abnormal extracellular

25 amyloid plaque that characterizes the brains of
victims of Alzheimer's disease (AD) (Haass and Selkoe,
1993). *In vitro* studies have shown that some of the
metal ions found in biological systems, i.e. Fe, Al
and Zn, can accelerate the aggregation process

30 dramatically. The presence of "pathological"
chaperones (Wisniewski and Frangione, 1992) and the
above listed metals (Mantyh et al., 1993; Fraser et
al., 1993) as proposed risk factors in Alzheimer's
disease, favor β-amyloid cascade aggregation. If the

35 interaction between the metal ion and the β-amyloid
can be interrupted or prevented, then metal-induced

aggregation can be reduced or eliminated. However,
just binding a mAb at this site might prevent the
metal-induced aggregation but would not allow normal
functioning of the protein.

5   It would therefore be useful to develop the
appropriate mAb with chaperone-like characteristics
directed to the appropriate epitope on the β-amyloid
molecule in order to prevent the accelerated metal-
induced aggregation.

10   In prion diseases it has been shown that the
pathologic "scrapie prion protein" propagates itself
by contacting normal prion protein and causing them to
unfold and flip from their usual conformation to the
scrapie prion protein state, a beta-pleated sheet.

15 This initiates a cascade effect. It would therefore
be useful to be able to interrupt the "scrapie-prion"
cascade.

         Further, it would be particularly useful to
be able to develop a mAb as needed that prevents the

20 aggregation of proteins/enzymes in vivo but that still
allows the proteins/enzymes to function even when
bound to the mAb.

         With the advent of recombinant DNA methods
for the production of proteins in bacteria methods are

25 ·needed to prevent aggregation during production and
harvesting. Chaperones have been shown to suppress
aggregation during the folding of a number of protein
in vitro and co-expression of chaperones can also
suppress formation of inclusion bodies by some foreign

30 proteins in vivo as described herein. However,
despite the use of chaperones, protein aggregation can
still occur during production and harvesting.

         Still further, it is not always possible to
isolate the appropriate chaperone for preventing

35 aggregation of a molecule and to utilize it as a
therapeutic. The availability of engineering and

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selecting mAbs and delivery systems for mAb makes it useful to develop specific mAb to serve as therapeutic chaperones.

5   **SUMMARY OF THE INVENTION AND ADVANTAGES**
              According to the present invention, a method is provided of selecting an anti-aggregation molecule such as a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics
10   the binding site of an antibody. These anti-aggregation molecules are able to bind to a native target molecule epitope with a high binding constant and are selected to be non-inhibitory to biological activity of the target molecule.
15   The present invention further provides a method of treating a protein aggregation disease by creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human form of the anti-aggregation molecule that
20   binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.
              The present invention also provides an expression vector comprising nucleic acid including a
25   sequence which encodes in expressible form the human form of the anti-aggregation molecule that binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.
30

              BRIEF DESCRIPTION OF THE DRAWINGS
              Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed
35   description when considered in connection with the accompanying drawings wherein:

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FIGURE 1 is a bar graph of the temperature-
dependence of enzymic activity of Carboxypeptidase A
(CPA); the residual enzymic activity of CPA after one
hour incubation at increasing temperatures was
5   measured using esterase substrate;

FIGURE 2 is a bar graph of the time course
of denaturation of Carboxypeptidase A after exposure
at 50°C; the residual esterase (□) and peptidase
10   (cross-hatch) enzymic activity of CPA was measured at
two intervals of incubation at 50°C; the amount of
residual soluble enzyme was determined by sandwich
ELISA (■);

FIGURE 3 is a bar graph of the enzymic
15   activity of Carboxypeptidase A retained after exposure
to 50°C for one hour in the presence of monoclonal
antibody CP_{10}; the immunocomplexation of CPA with
increasing amounts of CP_{10} was performed before
20   exposure at 50°C for one hour; the residual peptidase
(■) and esterase (□) enzymic activity of CPA was
measured;

FIGURE 4 is a bar graph of the effect of
25   epitope location on the maintenance of the enzymic
activity of heat-exposed Carboxypeptidase A;
increasing amounts of monoclonal antibodies CP_{10}
(cross-hatch) and CP_9 (□) and unrelated IgG (■) were
added to CPA before exposure to 50°C for one hour and
30   esterase enzymic activity was measured;

FIGURE 5 is a bar graph of the prevention of
aggregation of Carboxypeptidase A by monoclonal
antibody CP_{10}; aggregation of CPA, in the presence
35   (double cross-hatch bars) and in the absence (single
cross-hatch bars) of antibodies, was followed by

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determination of amount of mAb bound to coated CPA in
a competitive ELISA; the absorbance at 495 nm obtained
in the absence of added soluble CPA was set at 100%
for bound antibody; the soluble CPA, before heat
5   exposure, competes with the coated CPA for antibody
binding, leading to decrease in amount of antibody
bound (60%) (diagonal lines);

       FIGURE 6 is a bar graph showing thermal
10   aggregation of Carboxypeptidase A and its suppression
by monoclonal antibodies CP_{10} and CP_9; aggregation of
Carboxypeptidase A after exposure at 50°C for one hour
in the absence (☐) of monoclonal antibodies and in the
presence of CP_{10} (cross-hatch) and CP_9 (■) was followed
15   by determination of amount of antibody bound by
sandwich ELISA; maximum binding (100%) was considered
the amount of antibody bound to CPA before exposure to
aggregation conditions;

20   FIGURE 7 is a pair of graphs (A and B)
showing aggregation of β-amyloid (1-40) in the absence
(cross-hatch) and in the presence (☐) of monoclonal
antibodies AMY-33 (A) and 6F/3D (B) followed by ELISA;
(1) β-amyloid alone, (2) β-amyloid + 50 MM heparan
25   sulfate, (3) β-amyloid + 10^{-3}M $AlCl_3$; (4) β-amyloid +
10^{-3} M $ZnCl_2$;

       FIGURE 8 is a pair of graphs (A and B)
showing a Thioflavin T based fluorimetric assay of β-
30   amyloid aggregation (■) in the presence of mAb AMY-33
(☐) and an unrelated antibody (cross-hatch) with (A)
an emission spectra of Thioflavin T bound to fibrillar
β-amyloid peptide (upper curve) and in the presence of
antibody AMY-33 the immunocomplex with βA4 (lower
35   curve) and (B) shows an increase in Thioflavin T
fluorescence bound to β-amyloid peptide (■) as a

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function of incubation time of the peptide as measured
after three hours, three days and seven days wherein
immunocomplexation with mAb AMY-33 prevents the
increase in Thioflavin T fluorescence (□), while
5   unrelated antibody (cross-hatch) did not interfere
with its fluorescence; and

         FIGURE 9 is a bar graph showing aggregation
in the presence of various monoclonal antibodies to β-
10   amyloid peptide in the absence of soluble β-amyloid
(100%, maximal binding) and calculated by comparison
with the binding of the same monoclonal antibodies to
the residual soluble β-amyloid peptide remaining after
incubation in the absence of the respective antibody.
15

         DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT
         The present invention provides a method of
selecting monoclonal antibodies, genetically
20   engineered antibody fragments and small peptides which
mimic binding sites of the antibodies that prevent
aggregation of a native target molecule, which may
aggregate and is in general a protein, and yet do not
inhibit bioactivity when bound to the target molecule.
25   These anti-aggregation molecules with chaperone-like
activity are able to bind to a native target molecule
epitope with a high binding constant and yet are
selected to be non-inhibitory to the biological
activity of the target molecule when bound.
30   The method can include culturing an
appropriate host cell transformed with DNA encoding
the target molecule. The host cell chosen will
express the target molecule in aggregated form.
Examples of such cells are set forth in PCT published
35   international patent application 93/11248, 93/13200
and 94/08012. Alternatively, the appropriate

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recombinant target molecule can be purchased or a
naturally occurring molecule can be isolated or
purchased.

        The expressed target molecule is recovered
5   and denatured thereby also deaggregating the molecules
    if they are aggregated. The denatured target molecule
    is mixed with the presumptive anti-aggregation
    molecule such as a monoclonal antibody, genetically
    engineered antibody fragment or small peptide which
10 mimics an antibody binding site generally as set forth
    in PCT pending application 93/13200 and under
    conditions which allow for renaturing and for self-
    aggregation such as temperature, pH or interaction
    with other aggregation-inducing agents. Mixtures
15 which produce non-aggregated target molecules are
    selected. It is then determined if the nonaggregated
    target molecules are bioactive even in the presence
    of, and bound to, the presumptive anti-aggregation
    molecule.

20   In addition, the anti-aggregation molecule
    is screened for its ability to dissolve already
    aggregated proteins. The aggregated proteins are
    mixed with the anti-aggregation molecules under
    physiological conditions. Mixtures with non-
25 aggregated molecules are selected. It is then
    determined if the nonaggregated target molecules are
    bioactive even in the presence of, and bound to, the
    presumptive anti-aggregation molecule.

        The antibodies, or peptide mimicking the
30 binding site, bind to an epitope on the target
    molecule which is a region responsible for folding or
    aggregation. In addition the anti-aggregation
    molecule is selected only if it does not show immune
    cross reactivity with other proteins with proximity to
35 the target molecules under the same conditions
    employed in the bioactivity tests; that is, molecules

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which are found in the cell near the target molecule
or molecules with sequences similar to the target
molecules.

5   After the identification of the anti-
aggregation molecules has been completed, it is
possible in one embodiment to utilize two or more to
prevent or reverse aggregation. They can be used
concurrently to increase their chaperone-like effect,
if their respective target epitopes are not
10   overlapping and if, in binding to the target molecule,
they do not interfere with each other.

Bioactivity is tested as is appropriate for
the target molecule. For example, enzymatic activity
of the target molecule for its substrate can be
15   measured. Assays which measure *in vitro* enzymatic
bioactivity are well known to those skilled in the
art.

In one embodiment of the method, the target
molecule is β-amyloid and the monoclonal antibody is
20   an anti-β-amyloid monoclonal. Alternatively, a
genetically engineered antibody fragment as described
hereinbelow can be used or a small peptide which
mimics the antigen binding site on the target molecule
can be used. The antigen binding site of an antibody
25   can be determined as is known in the art as for
example from x-ray crystallography, DNA sequences or
the like.

The method has also been demonstrated with
carboxypeptidase A as set forth in the Examples
30   hereinbelow.

Other peptides or proteins with evidence of
self aggregation can also be used in the present
invention such as amylin (Young et al., 1994);
bombesin, caerulein, cholecystokinin octapeptide,
35   eledoisin, gastrin-related pentapeptide, gastrin
tetrapeptide, somatostatin (reduced), substance P; and

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peptide, luteinizing hormone releasing hormone,
somatostatin N-Tyr (Banks and Kastin, 1992), and prion
protein (PrP).

5   Once an appropriate monoclonal antibody with
chaperone-like activity is found or engineered or a
peptide with the appropriate configuration, the
present invention provides for its use therapeutically
to prevent or reduce protein aggregation *in vivo*. In
an embodiment, the prevention of β-amyloid aggregation
10   or prion-associated protein aggregation is undertaken.

· A method of treating a protein aggregation
disease intracellularly includes the steps of
preparing (Haber, 1992; Harlow & Lane, 1988) or
selecting an anti-aggregation molecule as set forth
15   herein, such as a monoclonal antibody, genetically
engineered monoclonal antibody fragment or peptide
that mimics the binding site of an antibody, that
binds to an aggregating protein which is the cause of
a disease and which prevents aggregation and yet
20   allows the protein to be bioactive even while bound.
This molecule can be referred to as an anti-
aggregation molecule with chaperone-like activity. An
expression vector is created comprising nucleic acid
including a sequence which encodes in expressible form
25   the anti-aggregation molecule. The expression vector
is then delivered to the patient.

In an embodiment a human monoclonal antibody
that binds to an aggregating protein and which
prevents aggregation is utilized. In a further
30   embodiment the monoclonal antibody (mAb) is an anti-β-
amyloid and is selected from the group consisting of
AMY-33 which recognizes an epitope spanning amino
acids 1-28 of β-amyloid, mAbs 6C6 and 10D5, which
recognize an epitope spanning the amino acid residues
35   1-16 of β-amyloid peptide, and mAbs 2H3 and 1C2,

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directed to the regions comprising peptides 1-12 and
13-28, respectively.

Work by Dueñas et al. (1994) and Marasco et
al. (1993) have shown that single chain monoclonal
5   antibodies are efficient for intracellular expression
in eukaryotic cells. The single chain monoclonal
antibody is composed of an immunoglobulin heavy chain
leader sequence and heavy and light chain variable
regions that are joined by an interchain linker. The
10 molecule is small, approximately 28 kDa with high-
affinity ligand-binding capability and minimal
assembly requirement. The antibody can be directed to
the relevant cellular compartment using classical
intracellular-trafficking signals. Marasco et al.
15 (1993) have shown that such antibodies are not toxic
to the cells and function when expressed in the cell.

The production of expression vectors is well
known to those skilled in the art. In a preferred
embodiment, the expression vector is constructed using
20 the methodology as set forth by Dueñas et al. (1994),
PCT pending application 94/11513. Methods not
explicitly set forth are performed as generally set
forth in Sambrook et al., *Molecular Cloning: A
Laboratory Manual*, Cold Springs Harbor Laboratory, New
25 York (1992), and in Ausubel et al., *Current Protocols
in Molecular Biology*, John Wiley and Sons, Baltimore,
Maryland (1989).

Such vectors are known or can be constructed
by those skilled in the art and should contain all
30 expression elements necessary to achieve the desired
transcription of the sequences. Other beneficial
characteristics can also be contained within the
vectors such as mechanisms for recovery of the nucleic
acids in a different form. Phagemids are a specific
35 example of such beneficial vectors because they can be
used either as plasmids or as bacteriophage vectors.

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Examples of other vectors include viruses such as
bacteriophages, baculoviruses and retroviruses, DNA
viruses, cosmids, plasmids, liposomes and other
recombination vectors. The vectors can also contain
5   elements for use in either procaryotic or eucaryotic
host systems. One of ordinary skill in the art will
know which host systems are compatible with a
particular vector.

The expression vector can be a virus.
10 Further the virus can be an RNA virus such as a
disabled retro virus or a retroviral shuttle vector.
The expression vector can also be vaccinia virus or an
adenovirus. The expression vector can also be a
plasmid. In a preferred embodiment wherein β-amyloid
15 is the targeted molecule the expression vector is
selected that is known to target the central nervous
system.

In the present invention, the expression
vector for use as a therapeutic agent comprises a
20 nucleic acid including at least one sequence which
encodes in expressible form an anti-aggregation
molecule, which molecule binds to an aggregating
protein that is the cause of a disease and which
prevents aggregation but does not interfere with
25 bioactivity. In an embodiment the expression vector
includes the sequence for a human monoclonal antibody
that is an anti-β-amyloid monoclonal antibody with
heparan-like characteristics. In a further preferred
embodiment, the expression vector includes the
30 sequence for the single chain monoclonal antibody of
the above anti-β-amyloid mAb.

A specific example of a DNA viral vector for
introducing and expressing recombinant sequences is
the adenovirus derived vector Adenop53TK. This vector
35 expresses a herpes virus thymidine kinase (TK) gene
for either positive or negative selection and an

expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector, as well as others that exhibit similar desired functions, can be used to

5   treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its

10   therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus such as antibiotic sensitivity. Negative selection is therefore a means by which infection can be controlled

15   because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur. Features that

20   limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are

25   useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a

30   single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of

35   infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be

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produced that are unable to spread laterally. This
characteristic can be useful if the desired purpose is
to introduce a specified gene into only a localized
number of targeted cells.

5   As described above, viruses are very
specialized infectious agents that have evolved, in
many cases, to elude host defense mechanisms.
Typically, viruses infect and propagate in specific
cell types. The targeting specificity of viral
10   vectors utilizes its natural specificity to
specifically target predetermined cell types and
thereby introduce a recombinant gene into the infected
cell. The vector to be used in the methods of the
invention will depend on desired cell type to be
15   targeted and will be known to those skilled in the
art. For example, if breast cancer is to be treated
then a vector specific for such epithelial cells would
be used. Likewise, if diseases or pathological
conditions of the nervous system are to be treated,
20   then a viral vector that is specific for neural cells
such as neurons, oligodendroglia and the like and
their precursors, preferably for the specific type of
hematopoietic cell, would be used.

Retroviral vectors can be constructed to
25   function either as infectious particles or to undergo
only a single initial round of infection. In the
former case, the genome of the virus is modified so
that it maintains all the necessary genes, regulatory
sequences and packaging signals to synthesize new
30   viral proteins and RNA. Once these molecules are
synthesized, the host cell packages the RNA into new
viral particles which are capable of undergoing
further rounds of infection. The vector's genome is
also engineered to encode and express the desired
35   recombinant gene. In the case of non-infectious viral
vectors, the vector genome is usually mutated to

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destroy the viral packaging signal that is required to
encapsulate the RNA into viral particles. Without
such a signal, any particles that are formed will not
contain a genome and therefore cannot proceed through
5   subsequent rounds of infection. The specific type of
vector will depend upon the intended application. The
actual vectors are also known and readily available
within the art or can be constructed by one skilled in
the art using well-known methodology as well as many
10   being commercially available.

        The expression vector containing the
sequence for the anti-aggregation molecule may be
administered to mammals, including humans, by any
route appropriate to the condition being treated and
15   in several ways. Suitable routes include oral,
rectal, nasal, topical, vaginal and parenteral. It
will be appreciated that the preferred route may vary
with, for example, the condition of the recipient and
the type of treatment envisaged.

20   If viral vectors are used, for example, the
procedure can take advantage of their target
specificity and consequently, do not have to be
administered locally at the diseased site. However,
local administration can provide a quicker and more
25   effective treatment, administration can also be
performed by, for example, intravenous or subcutaneous
injection into the subject. Injection of the viral
vectors into spinal fluid can also be used as a mode
of administration, especially in the case of neuro-
30   degenerative diseases. Following injection, the viral
vectors will circulate until they recognize host cells
with the appropriate target specificity for infection.
Alternatively, the method as set forth by Tuomanen et
al. (1993) can be used.

35   The vectors can be introduced into cells or
tissues by any one of a variety of known methods

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within the art. Such methods can be found described
in Sambrook et al. and Ausubel et al., and include,
for example, stable or transient transfection,
lipofection, electroporation and infection with
5   recombinant viral vectors. Introduction of nucleic
acids by infection offers several advantages over the
other listed methods as indicated herein. Higher
efficiency can be obtained due to their infectious
nature. Moreover, viruses are very specialized and
10   typically infect and propagate in specific cell types.
Thus, their natural specificity can be used to target
the vectors to specific cell types *in vivo* or within a
tissue or mixed culture of cells. Viral vectors can
also be modified with specific receptors or ligands to
15   alter target specificity through receptor mediated
events.

An alternate mode of administration of the
vector can be by direct inoculation locally at the
site of the disease or pathological condition or by
20   inoculation into the vascular system supplying the
site with nutrients. Local administration is
advantageous because there is no dilution effect and,
therefore, a smaller dose is required to achieve
expression in a majority of the targeted cells.
25   Additionally, local inoculation can alleviate the
targeting requirement required with other forms of
administration since a vector can be used that infects
all cells in the inoculated area. If expression is
desired in only a specific subset of cells within the
30   inoculated area, then promoter and regulatory elements
that are specific for the desired subset can be used
to accomplish this goal. Such non-targeting vectors
can be, for example, viral vectors, viral genome,
plasmids, phagemids and the like. Transfection
35   vehicles such as *liposomes* can also be used to
introduce the non-viral vectors described above into

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recipient cells within the inoculated area. Such
transfection vehicles are known by one skilled within
the art.

The expression vector of the present
5   invention may be administered to the patient alone or
in combination with liposomes or other delivery
molecules. The expression vector is administered and
dosed in accordance with good medical practice, taking
into account the clinical condition of the individual
10   patient, the site and method of administration,
scheduling of administration, and other factors known
to medical practitioners. The "effective amount" for
purposes herein is thus determined by such
considerations as are known in the art. The amount
15   must be effective to achieve in the treated patients a
reduction in protein aggregation and may also include
but is not limited to improved survival rate, more
rapid recovery, or improvement or elimination of
symptoms and are selected as appropriate measures by
20   those skilled in the art.

While it is possible for the expression
vector containing the sequence for the anti-
aggregation molecule to be administered alone, it is
preferable to present it as a pharmaceutical
25   formulation. The formulations of the present
invention comprise at least one active ingredient: the
monoclonal antibody or expression vector together with
one or more pharmaceutically acceptable carriers and
optionally other therapeutic ingredients. The
30   carrier(s) must be acceptable in the sense of being
compatible with the other ingredients of the
formulation and not deleterious to the recipients.
The carriers must also be selected so as not to
interfere with the activity of the active ingredient.
35   Further the present invention also provides
a method of improving solubility and yields in

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production of recombinant proteins, particularly in
blocking the formation of inclusion bodies in the host
cells for the *in vitro* production of recombinant
proteins. The present invention provides an anti-
5   aggregation molecule that suppresses the kinetics of
aggregate formation while still encouraging formation
of native protein structure, and favors the desired
folding reaction thereby improving yields of the
recombinant product. A genetically engineered
10   antibody fragment as described herein can be used or a
small peptide which mimics the antigen binding site on
the target molecule can be used.

In a preferred embodiment a single-chain
antibody (single-chain variable region; SCFVS) in
15   which the heavy and light chain variable domains is
engineered as a single polypeptide (Dueñas et al.,
1994; Marasco et al., 1993) and delivered to the
cells, either directly by methods described herein
above or in an appropriate expression vector as
20   described herein. Utilizing an expression vector, the
expression of the antibody and of the *desired gene*
product, in general an overexpressed recombinant
product, are effected by introducing the either two
vectors one encoding the gene for the recombinant
25   produce and one for the antibody or a vector
containing both into the host cell. The result is a
small, approximately 28 KDa molecule with high-
affinity ligand-binding capability and minimal
assembly requirement that is co-synthesized with the
30   recombinant protein product in the host cell. The
antibody can be directed to the relevant cellular
compartment using classical intracellular-trafficking
signals. Functional studies have illustrated that
single chain antibodies are able to fold and assemble
35   correctly in the cytoplasm. The *co-expression of the*
antibody and the recombinant product increases

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intracellular production of the fusion proteins and
prevents the formation of the inclusion bodies and/or
facilitates extracellular protein secretion. The
fusion proteins obtained, in addition to better
5   solubility and yield, exhibit increased thermal and
proteolytic resistance.

The availability of monoclonal antibodies
which bind to a specific antigen at distinct and well
defined sites has led to a better understanding of the
10   effects of highly specific enzyme-antibody
interactions on the enzyme behavior. By appropriate
selection it has been possible to isolate those
antibodies that are non-inhibitory to biological
activity of the enzyme and bind at "strategic
15   locations" on the antigen molecule, resulting in a
considerable stabilization effect of the enzyme
conformation. Moreover, such monoclonal antibodies,
by selection using the present invention, prove to
have a chaperone-like activity leading to a
20   considerable refolding effect on the enzyme which was
already partially heat denatured. In addition, the
use of engineered monoclonal antibodies and their
fragments, as well as peptides which mimic the binding
site for the antigen on the antibody can be used in
25   the present invention.

In the model test system shown in Example 1
herein below, Carboxypeptidase A (CPA) shows a
decrease in solubility with an increase in
temperature, accompanied by loss of enzymic activity
30   and conformational changes leading to its aggregation.
In the Example, the suppression of enzyme aggregation
via its interaction with two monoclonal antibodies
raised against native protein was investigated. ELISA
measurements and determination of residual enzymic
35   activity, as a probe of the native structure, were
used to monitor the protein aggregation process. The

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studied monoclonal antibodies are non-inhibitory to
the biological activity of the antigen or target
molecule, bind on the strategic position on the
molecule and proved to have a chaperone-like activity
5   in the prevention of protein aggregation. The
antibodies effect on the inhibition of aggregation was
found to be related to the location of the antigenic
site of each antibody. Based on the experimental
data, the formation of the immunocomplexes will
10   provide a general and convenient method for
suppression of aggregation and stabilization of the
target molecules without affecting the biological
properties of the given target molecule. The present
invention uses genetically engineered antibodies
15   obtained from such selected antibodies as protecting
agents of *in vivo* aggregation of their antigen,
leading to production of a soluble and stabilized
protein.
         Protein aggregation is of major importance
20   that extends into mechanisms of human diseases and
fundamental aspects of protein folding, expression and
function. Data in the literature (De Young et al.,
1993; Wetzel, 1994; Wetzel, 1991) suggests that
aggregation is non-specific in the sense that addition
25   of other proteins can influence the extent of
aggregation of a certain protein. However, the
specificity can be related to a particular residue or
group of residues which play a special role in the
folding-related aggregation of a polypeptide (Silen
30   and Agard, 1989; Zhu et al. 1989; Winter et al., 1994;
Brems 1988). The identification of such classes of
sequences that play a role in the folding-unfolding
and/or solubilization-aggregation provides the basis
of the present invention for the prevention of
35   aggregation.

Stabilization procedures based on protein-protein recognition processes, fundamental to biology, have been previously investigated (Chothia and Janin, 1975; Jaenicke, 1991). Introduction of molecular

5 chaperones which enable folding and stabilization of unrelated proteins appears to be tailored to prevent misfolding and aggregation at an early stage during folding. However, the central problem remaining in *in vivo* folding is how to efficiently prevent aggregation

10 without blocking the forward pathway of correct folding and biological activity of the native state (Ellis et al. 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993).

The availability of monoclonal antibodies

15 (mAbs) led to a better understanding of the effect of highly specific antigen-antibody interactions on the antigen or target molecule behavior. The complementary conformation between the interacting regions of the antibody with its antigen confers the

20 high specificity and stability to the immunocomplex formed (Goldberg, 1991). Properly selected monoclonal antibodies, unlike the ubiquitous nature of the chaperones, bind to a specific antigen at a distinct and preselected antigenic site without interfering in

25 the biological activity of the antigen and assist in antigen refolding (Blond and Goldberg, 1987; Carslon and Yarmush, 1992; Solomon and Schwartz, 1995).

The present invention utilized the effect of immunocomplexation in the suppression of antigen

30 aggregation using as a model system the interaction of Carboxypeptidase A (CPA) and its monoclonal antibodies. CPA occupies a prominent position in the literature of metalloenzymes, being a well-characterized zinc exopeptidase that exhibits both

35 peptidase and esterase activity (Vallee and Galdes, 1984). A large number of mAbs were prepared by the

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applicant towards native enzymes (Solomon et al. 1984) and their properties were investigated. Some of these antibodies bind to the enzyme with a relatively high binding constant, remote from its active site and
5   assist in refolding of already heat denatured enzyme (Solomon and Schwartz, 1995). ELISA measurements and determination of residual enzymic activity as a probe of native structure are used to monitor the effect of two different mAbs, namely CP_{10} and CP_9 on the
10   inhibition of CPA aggregation.

        Further, as shown in Examples 2 and 3 herein below, in suppressing β-amyloid aggregation, the monoclonal antibodies AMY-33 and 6F/3D, which recognized different epitopes of the β-amyloid peptide
15   chain, exhibited a selective chaperone-like activity. The immunocomplex of mAb AMY-33 + βA4 not only prevented self-aggregation of β-amyloid peptide but also the aggregation that was induced in the presence of heparan sulfate, which is thought to affect only
20   the aggregation of preexisting amyloid fibers (Talafous et al., 1994). The inhibitory effect was related to the localization of the antibody-binding sites and to the nature of the aggregating agents. The results of negative-staining electron microscopy
25   revealed that even at low concentrations of mAb AMY-33 only amorphous aggregates are formed. The ELISA measurements indicated that increasing the concentration of mAb AMY-33 to equimolar antigen/antibody ratios maintained β-amyloid peptide
30   solubility. The diffuse and amorphous conglomerates of Aβ deposits that were not detectable by thioflavin T fluorimetry or Congo Red staining are not supposed to be associated with neuritic pathology (Levine, 1993).

35   Because β-amyloid peptide has been shown to be physiologically produced in a soluble form in

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normal individuals (Seubert et al., 1992; Shoji et
al., 1992), the aggregation of soluble β-amyloid
peptide into insoluble amyloid fibrils is believed to
be a crucial step in the pathogenesis of Alzheimer's

5   disease.   Therefore, to reduce or eliminate the extent
of pathological protein depositions in the brain, much
effort has been focused on developing potent and
selective inhibitors of β-amyloid aggregation (Synder
et al., 1994; Tomiyama et al. 1994; Schwarzman et al.,

10   1994).   Preparing mAbs against "aggregating epitopes,"
identified as sequences related to the sites where
protein aggregation is initiated, thereby provides a
tool for preventing the phenomenon of protein
aggregation.   Applicant has shown that appropriate

15   mAbs interact at strategic protein-folding-initiation
sites, leading to a considerable refolding effect of
the already clustered epitopes.

        The mAb AMY-33 did not exhibit a similar
inhibitory effect on metal-induced amyloid

20   aggregation.   The slight interference with Zn^{2+}-induced
β-amyloid peptide aggregation that occurred using mAb
6F/3D may be due to the partial solvation effect of
already aggregated β-peptide.

        In experiments with additional monoclonal

25   antibodies, mAbs 6C6 and 10D5, which recognize an
epitope spanning the amino acid residues 1-16 of
β-amyloid peptide, inhibited the formation of
β-amyloid by 90% when compared with aggregation
occurring in the absence of the respective antibodies.

30   mAb AMY-33, which recognizes another epitope located
within residues 1-28, affected the self-aggregation of
β-peptide to a lower extent of approximately 40% in
the same set of experiments.   The antibodies, 2H3 and
1C2, directed to the regions comprising peptides 1-12

35   and 13-28, respectively, had a considerably lower
effect on *in vitro* amyloid formation.

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The above discussion provides a factual basis for the use of monoclonal antibodies and genetically engineered antibody fragments as therapeutics for the prevention of protein
5   aggregation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES
10   METHODS AND REAGENTS

Carboxypeptidase A (CPA)
            CPA was obtained as an aqueous crystalline suspension (Sigma Chemical Co., St. Louis, MO). The
15 crystals were washed with double-distilled water, centrifuged, and dissolved in 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278 nm.
20

Determination of CPA Enzymatic Activity
            The enzymatic activities of CPA and its immunocomplexes were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine
25 as peptidase substrate or hippuryl-DL-β-phenyllactic acid as esterase substrate in 0.5 M NaCl/0.05 M Tris-Hcl, pH 7.5, (Solomon et al, 1989).

Amyloid
30   Amyloid peptides, Aβ 1-40 (Cat. No. A-5813) and Aβ 1-28 (Cat. No. A-1084) corresponding to amino acids 1-40 and 1-28 of Aβ respectively, were purchased from Sigma Chemical Co., St. Louis, MO, USA.
            Amyloid solutions were prepared by
35 dissolving the peptides in water at concentration of

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10 mg/ml. The stock solution was stored in aliquots
at -20°C.

Aggregating agents

5   Heparan sulfate (Cat. No. H 5393) was
purchased from Sigma Chemical Co., St. Louis, MO, USA.
Stock solutions of metal chlorides were made up from
dry salts at concentration of 1mM in TRIS pH 7.4.

10   Monoclonal Antibody Production
         In general, monoclonal antibodies may be
prepared against a synthetic peptide based on the
sequence, or prepared recombinantly by cloning
techniques or the natural gene product and/or portions
15   thereof may be isolated and used as the immunogen.
Such proteins or peptides can be used to produce
monoclonals by standard production technology well
known to those skilled in the art as further described
generally in Harlow and Lane, *Antibodies: A Laboratory*
20   *Manual*, Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY, 1988, Milstein (1980) and Borrebaeck,
Antibody Engineering - A Practical Guide, W.M. Freeman
and Co., 1992. Briefly, mouse monoclonal antibodies
are prepared by hyperimmunization of an appropriate
25   donor with the protein or peptide fragment, generally
a mouse, and isolation of splenic antibody producing
cells. These cells are fused to a cell having
immortality, such as a myeloma cell, to provide a
fused cell hybrid which has immortality and secretes
30   the required antibody. The cells are then cultured,
in bulk, and the monoclonal antibodies harvested from
the culture media for use.
         The harvested monoclonal antibody can be
bound to a solid support substrate or conjugated with
35   a detectable moiety or be both bound and conjugated as
is well known in the art. For a general discussion of

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conjugation of fluorescent or enzymatic moieties see
Johnstone & Thorpe, *Immunochemistry in Practice*,
Blackwell Scientific Publications, Oxford, 1982. The
binding of antibodies to a solid support substrate is
5   also well known in the art. (see for a general
discussion Harlow & Lane *Antibodies: A Laboratory
Manual*, Cold Spring Harbor Laboratory Publications,
New York, 1988) The detectable moieties contemplated
with the present invention can include, but are not
10   limited to, fluorescent, metallic, enzymatic and
radioactive markers such as biotin, gold, ferritin,
alkaline phosphatase, β-galactosidase, peroxidase,
urease, fluorescein, rhodamine, tritium, ^{14}C and
iodination.
15   Alternatively, commercially available
antibodies can be used. α-Human β-amyloid 6F/3D was
obtained from ACCURATE Chemical and Scientific Corp.
(Westbury, NY, USA). mAb AMY 33 was purchased from
ZYMED San Francisco, CA, USA. A polyclonal, affinity
20   purified rabbit IgG obtained against the synthetic
Alzheimer β-amyloid (Cat. No. 1381431) was purchased
from Boehringer-Mannheim, GmbH, Germany. Another four
monoclonal antibodies called 6C6 and 10D5, raised
against peptide 1-28 of β-amyloid, 2H3 and 1C2 raised
25   against peptides 1-12 and 13-28, respectively, were
generously provided by Dr. D. Schenk, Athena
Neuroscience, San Francisco, CA.

<u>Purification and characterization of anti-CPA mAbs</u>
30   The monoclonal antibodies, CP-10, CP-9,
which interact with CPA at high binding constants,
were selected for further study. The preparation and
characterization of the monoclonal antibodies CP_{10} and
CP_9 (chosen for the present study) were previously
35   described (Solomon et al., 1989; Solomon and Balas,
1991).

These antibodies were isolated and purified
by affinity chromatography on protein A-Sepharose from
the corresponding ascites fluids according to Harlow
and Lane.

Protocol for determining effect of monoclonal antibody
binding on CPA activity

CPA (1 mg/ml) was incubated at 50°C in the
absence and in the presence of increasing amounts of
mAbs CP_{10} and CP_9 (100 μl in PBS) ranged between 0-2
molar ratio antibody/CPA. The enzymic activities of
the immunocomplexes formed were measured as described
herein above. Data related in percentage, 100% being
considered the enzymic activity of CPA before
denaturation.

ELISA Tests for CPA Studies

The antigen-coating solutions (100 μl
containing native CPA (10-25μl/ml) in PBS, pH 7.4,
were incubated overnight at 4°C in a polystyrene ELISA
plate (Costar, Cambridge, MA). Diluted ascites fluid
(0.1 ml) containing the desired mAb (1:2000 to
1:18,000 v/v in PBS) was added and incubated at 37°C
for 1 hour. The amount of bound mAb was determined
with β-galactosidase-linked F(ab)$_2$ fragments of sheep
anti-mouse IgG (Amersham International, UK).

The quantitation of the amount of aggregated
CPA during denaturation at 50°C was determined by
competitive and sandwich ELISA, as follows:

Competitive ELISA assays for CPA Studies

CPA (10 μl/ml of PBS) was adsorbed onto
ELISA plates overnight at 4°C, the remaining active
groups on the plate being blocked with non-fat milk.
To the soluble CPA (200 ng in 10 μl PBS), incubated
for one hour at 50°C, the mAb CP_{10} (molar ratio 1:1

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Ab/CPA) was added and allowed to interact with the
remaining soluble CPA for one hour at 37°C. In
parallel, the mAb was added to the CPA solutions
before exposure at 50°C for one hour. After
5 incubation, the CPA preparations were removed by
centrifugation at 15,000 rpm for 15 minutes and
applied on the ELISA plates coated with CPA. The
antibody which did not bind to soluble CPA in the
reaction mixture will bind to the coated CPA; the
10 amount of antibody bound to the coated antigen will be
conversely proportional to the extent of CPA
aggregation and determined using α-mouse antibodies
labeled with horseradish peroxidase (HRP). The color
developed by HRP (O-phenylenediamine (OPD) as
15 substrate) was measured at OD_{495} using an ELISA plate
reader. The amount of antibody bound on the coated
CPA in the absence of soluble CPA was considered as
100%.

20 <u>Sandwich ELISA for CPA Studies</u>
         The ELISA plates were coated with rabbit
polyclonal antibodies raised against CPA (1μl/well) by
incubation at 37°C for two hours. The residual active
groups were blocked by non-fat milk. Soluble CPA (200
25 ng in 10μl PBS) was exposed to 50°C for one hour and
the aggregated CPA was removed by centrifugation at
15,000 g for 15 minutes. The residual soluble CPA was
incubated for another one hour at 37°C with mAb CP_{10}
and mAb CP_9 at various molar ratio antibody/antigen.
30 In another set of experiments, the mAbs were added to
the reaction mixtures before incubation at 50°C and
then exposed for one hour at 50°C. After the
incubation period, all the immunocomplexed CPA
preparations were centrifuged and added to the ELISA
35 plate, previously coated with polyclonal CPA
antibodies, for 12 hours at 4°C. The amount of mAb

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bound, determined as described above, will be
proportional to the amount of soluble CPA which
remained after exposure to aggregation conditions.
The results are presented in percentages, 100% being
5   the maximal absorbance obtained before CPA heat
treatment.

All data presented are the mean of
triplicate determinations. The standard deviation of
the intra-assay and interassays were less than 5% in
10   all cases.

Amyloid ELISA Assays

The ELISA plates were coated with rabbit
polyclonal antibodies (Boeringer-Mannheim) raised
15   against synthetic β-amyloid (1-40) (Sigma) (100
ng/well) via covalent attachment to epoxy-coated ELISA
plates by incubation at 4°C for 16 hours. (Eupergit-C
containing epoxy groups, Rohm GmbH, Darmstadt,
Germany, using beads or paper as the solid phase
20   Solomon, et al., 1992; Solomon et al., 1993). The
residual epoxy groups were blocked by non-fat milk.

The reaction mixtures containing aqueous
solution of β-amyloid (100 ng/ml), heparan sulfate (50
mM) and/or chloride metal solutions (10^{-3}M at pH 6.5),
25   was first incubated at 37°C for three hours. The
aggregated β-amyloid preparations were removed by
centrifugation at 15,000 g for 15 minutes. The
residual soluble β-amyloid was incubated for another
one hour at 37°C with mAbs AMY 33 and/or 6F3D at equal
30   molar ratio antibody/antigen. In another set of
experiments, the mAbs were added to the reaction
mixtures before incubation at 37°C and then incubated
together for 3 hours at 37°C.

After the incubation period, the
35   immunocomplexed amyloid preparations were added to the
ELISA plates, previously coated with polyclonal anti-

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amyloid antibodies. The amount of mAb bound will be
proportional to the amount of soluble amyloid which
remained after exposure to aggregation conditions.

The amount of bound antibody was determined
5   using α-mouse second antibodies labeled with
horseradish peroxidase (HRP). The enzyme activity of
HRP is directly proportional with the amount of
residual amyloid bound to rabbit polyclonal
antibodies. The enzyme activity of HRP was measured
10   using O-phenylenediamine (OPD) as substrate. The
color developed was measured at A_{495} using an ELISA
reader. Data represent the mean of triplicate
determinations. The standard deviation of the intra-
assay and interassays were less than 5% in all cases.
15

Competitive enzyme-linked immunosorbent assay (ELISA)

β-Amyloid peptide (50 ng/well in PBS) was
covalently bound to epoxy-coated ELISA plates
overnight at 4°C. The remaining active groups on the
20   plate were blocked with skim milk.

The reaction mixture containing soluble
β-amyloid peptide (50 ng in 10 μl PBS) was first
incubated for three hours at 37°C. Each of the mAbs
was added and allowed to interact with the remaining
25   soluble β-amyloid peptide for one hour at 37°C. The
amount of each antibody was sufficient to bind to all
soluble β-amyloid peptide (50 ng) before first
incubation at 37°C. In parallel assays, the same
amounts of mAbs were added to the β-amyloid peptide
30   solutions before the first incubation at 37°C for
three hours. After the second incubation, the
β-amyloid preparations centrifuged at 15,000 x g for
15 minutes and the soluble reaction mixture was
applied onto the ELISA plates previously coated with
35   β-amyloid peptide.

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The antibody that does not bind to the
insoluble β-peptide removed by centrifugation will
bind to the β-amyloid peptide coated on the ELISA
plate. The amount of antibody bound to the β-amyloid
5   peptide coated plate will be inversely proportional to
the amount of residual soluble β-amyloid peptide. The
amount of bound antibody was detected using α-mouse
antibodies labeled with horseradish peroxidase (HRP).
Degradation of the O-phenylenediamine (OPD) substrate
10   by HRP was monitored at OD_{495} using an ELISA reader.
The maximal amount of antibody necessary to bind all
the coated β-amyloid peptide in the absence of
β-soluble peptide was determined for each mAb and was
considered as 100% of mAb bound. The data represent
15   the mean of five replicates.

β-Amyloid Peptide Aggregation and Immunocomplexation
Synthetic β-amyloid peptide 1-40 (Aβ 1-40)
was obtained from Sigma Chemical Co., St. Louis,
20   Missouri, USA. Reaction mixture tubes containing 200
μl of an aqueous solution of β-amyloid peptide (10
mg/ml) pH 6.7 were incubated for three hours at 37°C.
Aggregated β-amyloid samples were removed by
centrifugation at 15,000 x g for 15 minutes. Aliquots
25   of residual soluble β-amyloid peptide were then
incubated for another 60 minutes with increasing
amounts of the appropriate mAbs to produce totally
immunocomplexed β-amyloid peptide. In another set of
experiments, mAbs at equimolar antibody/antigen
30   concentrations were added to the reaction mixtures
before the first incubation period of three hours at
37°C.

Electron Microscopy.
35   Negatively stained amyloid fibrils were
prepared by floating carbon-coated grids on aqueous

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peptide solutions (1-2 mg/ml) and air drying. Fibrils
of β-amyloid, either alone or immunocomplexed to mAb
AMY-33 (molar ratio 4:1) for three hours at 37°C, were
negatively stained with aqueous uranyl acetate (2%
5   wt/vol) and then visualized using a JEOL-1200 Ex
electron microscope operated at 80/KV, using a
magnification of 25,000.

Fluorimetry.

10   Fluorimetric analysis of soluble β-amyloid
peptide and the immunocomplex with AMY 33 (molar ratio
4:1) stained with Thioflavin T (Sigma Chemical Co.,
St. Louis, MO., USA) was performed by standard method
(Levine, 1993). Fluorescence was measured using a
15   Perkin-Elmer LS-50 fluorimeter at λ_{ex} = 482 nm. The
aggregation reaction was followed for seven days at
37°C.

EXAMPLE 1
20   CPA Model System
            Aggregation of heat denatured CPA was
followed by determination of the residual enzymic
activity of CPA using esterase and peptidase
substrates. CPA (1 mg/ml) was incubated at various
25   temperatures for one hour, and residual enzymic
activity was determined. The temperature of 50°C was
chosen for further study. At this temperature, mAbs
studied keep all their immunological activity
(unpublished data). Effect of immunocomplexation of
30   CPA with its mAbs was monitored by: (1) Determination
of enzymic activity and (2) ELISA measurements as
described herein above.
            Monoclonal antibodies raised against native
antigens proved to be powerful tools in identification
35   and characterization of folding steps by recognition
of incompletely folded antigens (Hendrick and Hartl,

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1993). The selected antibodies might interact at
sites where protein unfolding is initiated, thereby
stabilizing the protein and suppressing further
aggregation.

5   The main difference between mAbs and
molecular chaperones is that the latter does not bind
to native proteins and is capable of interacting with
many different polypeptide chains without exhibiting
an apparent sequence preference (Goloubinof et al.,
10   1989). Moreover, chaperones suppress aggregation but
do not redissolve aggregate proteins already present.
        The aggregation of CPA and loss of its
enzymic activity was found to be dependent on the
temperature and the time of incubation (Figures 1, 2).
15   Esterase activity seems to be more affected at higher
temperature than peptidase activity, indicating that
these activities follow different reaction mechanisms
(Figure 2). These data are compatible with
applicant's previous results (Solomon et al., 1989;
20   Solomon and Balas, 1991), as well as with the findings
of Vallee and his collaborators (1969), who postulated
that the active site of CPA consists of non-identical
but interacting binding sites for peptides and ester
substrates. As shown in Figure 2, the immunological
25   recognition of partially heat denatured enzyme is
better conserved than its residual enzymic activity.
        The inhibition of CPA aggregation, induced
by incubation at 50°C for one hour by its interaction
with two mAbs, CP_9 and CP_{10}, was followed by measuring
30   the peptidase and esterase enzymic activities (Figure
3). The two mAbs, CP_{10} and CP_9 were chosen for this
study on the basis of previous data regarding their
effect on the enzyme behavior (Solomon and Schwartz,
1995; Solomon et al., 1989; Solomon and Balas, 1991).
35   The protection of enzymic activity of heated CPA was
dependent on the amount of antibody added to the

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enzyme and a molar ratio of 1:1 antibody/enzyme was
sufficient for the maximum protection effect. The
peptidase activity of the $CPA-CP_{10}$ complex was
maintained at 90% of its initial activity in the
5   presence of mAb CP_{10}. The protective effect of mAbs on
CPA activity during heat denaturation was found to be
related to the location of the antigenic site of each
antibody (Figure 4). Even a great excess of unrelated
antibody did not assist in maintaining CPA activity.
10   Increase in preservation of enzyme activity can be
reached, however, in the presence of a pair of two
antibodies. This effect seems to be the result of a
"locking" of the conformation caused by simultaneous
interaction with two different antibodies at two
15   distinct epitopes (Solomon and Balas, 1991).

The amount of aggregated CPA was quantitated
by ELISA measurements. Disappearance of CPA, as a
result of its aggregation during incubation for one
hour at 50°C, was followed by a competitive ELISA
20   assay (Figure 5) and a sandwich assay (Figure 6). The
mAb, CP_{10}, maintained 100% of the CPA activity in
solution during heating for one hour at 50°C (Figure
6); CP_9 provided a slight effect on CPA protection at
50°C. Both antibodies prevent the aggregation of CPA,
25   similar to the data shown in Figure 4, recognizing
"key positions" on the molecule responsible for heat
denaturation and aggregation of CPA.

The biological activity of the enzyme seems
to be more sensitive to high temperatures than the
30   insolubilization process. Subtle heat-induced
conformational changes occurring in CPA molecules are
reflected by change in enzymic activity, even before
transition between native-molten globule conformation-
aggregated states occurred. These findings are in
35   contradiction to previous suggestions that the

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biological function of a protein does not necessarily require fully folded protein (Hattori et al., 1993).

The antigen binding site of mAb CP_{10} (previously named CP_{100}) was identified as one of the immunodominant regions of the enzyme, localized on the surface of the molecule between amino acids 209-218 (Solomon et al. 1989). The localization of the epitope recognized by CP_9 has not yet been clarified, but it does not interfere with the mAb CP_{10} during simultaneous binding to CPA molecule, as suggested by additivity measurements (Solomon and Balas, 1991).

Similar effects in suppression of antigen aggregation were obtained after immunocomplexation of horseradish peroxidase.

The data available in the literature of antibody preparation suggests that for practically all antigens it should be possible to prepare monoclonal antibodies and by means of the present invention it will be possible to select those monoclonal antibodies which bind with high affinity without affecting the target molecule activity. Moreover, mAbs like the majority of immunoglobulins, are robust molecules and survive in a variety of environments, including high temperatures, low pH, denaturing agents. Formation of such immunocomplexes should provide a general and convenient method for suppression of aggregation and stabilization of their antigen without affecting the biological properties of the given antigen as shown also in the following Examples.

EXAMPLE 2

Amyloid System

This example investigates the immunocomplexation effect on the *in vitro* aggregation of β-amyloid. Aggregation of β-amyloid was found to be dependent on the pH, peptide concentration,

temperature and time of incubation (Burdick et al.,
1992). In applicant's experiments, the aggregation of
β-amyloid was performed by incubation of an aqueous
solution of βA4 (10 mg/ml) for three hours at 37°C.

5   The β-amyloid aggregation was followed by ELISA
measurements using two different commercially
available monoclonal antibodies raised against β-
amyloid: α-human β-amyloid 6F/3D obtained from
Accurate Chemical and Scientific Corp, Westbury, N.J.

10   USA, and mAb AMY 33 (Stern et al., 1990), purchased
from Zymed, San Francisco, CA, USA, raised against
peptides 8-17 and 1-28, respectively, of the β-
amyloid.

        The addition of the antibodies was made

15   before or after exposure of synthetic β-amyloid to the
aggregation process (Figs. 7A, B). The aggregation of
the β-amyloid was performed in the presence of heparan
sulfate and/or metal ions, such as Zn^{2+} and Al^{3+}. The
antibody AMY-33, which recognizes an epitope spanning

20   the amino acids 1-28 of the β-amyloid sequence,
inhibits the β-amyloid aggregation occurring in the
presence or absence of heparan sulfate (Figure 7A).
Any significant effect on metal-induced amyloid
aggregation was observed under the same experimental

25   conditions. The mAb 6F/3D, recognizing an epitope
located in amino acids 8-17 of β-amyloid, interferes
with Zn^{2+}-induced aggregation, showing a partial
solubilization effect on already aggregated β-amyloid,
but has no effect on other aggregating agents (Figure

30   7B).

        Metals, such as Zn^{2+} and Al^{3+}, have been
proposed as risk factors for Alzheimer's disease
development (Mantyh et al., 1993; Frederickson, 1989;
McLachlan et al., 1991). The aggregation of βA4

35   induced by aluminum is distinguishable from that
induced by Zn in terms of role, extent, pH and

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temperature dependence (Mantyh et al. 1993). Although
the precise site of interaction of metal ions and βA4
is not clarified, several residues in βA4 are
candidates for metal binding. The βA4 histidine

5   residues (His_{13}-His_{14}) may be implicated in fibril
formation and it is conceivable that at least H_{14}
remains available for intermolecular electrostatic
interactions between anti-parallel chains (Talfous et
al., 1994). The site defined by Val_{12}-His_{13}-His_{14}-Glu_{15}-

10 Lys_{16}-Leu_{17} has been identified as a sequence containing
a heparan sulfate binding domain (Fraser et al., 1992)
and His_{13} and Lys_{16} are supposed to provide the cationic
binding sites being exposed on the same face of the
peptide β sheet (Talafous et al. 1994).

15   Binding of mAb AMY-33 to βA4 prevents self-
aggregation of the β-amyloid. This antibody prevents
intramolecular aggregation occurring in the presence
of heparan sulfate, which is supposed to affect only
the aggregation of preexisting amyloid fibers (Fraser

20 et al., 1992). Inhibition of β-amyloid aggregation in
the presence of mAb 6F/3D was partially effective only
in the presence of Zn^{2+}.

EXAMPLE 3

25 Amyloid Electron Microscopy and Fluorimetric Studies
            Electron microscopy of negatively stained
β-amyloid and its immunocomplex with mAb AMY-33
revealed that even at a low peptide/antibody ratio,
fibrillar β-amyloid was converted to an amorphous

30 state. Thioflavin T, a suitable probe for detecting
the fibrillar aggregation of β-amyloid peptide,
confirmed the electron microscopy results. Dilution
of the peptide directly from water into dye-containing
buffer had no effect on dye fluorescence. In the

35 presence of aggregated Aβ4 (1-40), however, a change
occurred in the excitation spectrum of Thioflavin T,

manifested as a new peak at 450 nm that was not seen
with the free dye (Figs 8A, B.) Aggregated β-amyloid
peptide enhances the fluorescence emission of
Thioflavin T at 482 nm as a function of
5   incubation-time. Adding mAb AMY-33 (4:1) to the Aβ
1-40 solution before exposure to 37°C prevented the
increase in fluorescence at 482 nm, whereas the
addition of unrelated antibody did not interfere with
the fluorescence peaks.
10

EXAMPLE 4

Comparative Studies of Monoclonal Antibodies

       Monoclonal antibodies were added to the
reaction mixture before or after incubation of
15   synthetic β-amyloid peptide at 37°C for three hours.
The results shown in Figure 9 indicate that mAbs 6C6
and 10D5, which recognize an epitope spanning the
amino acid residues 1-16 of β-amyloid peptide,
inhibited the formation of β-amyloid by 90% when
20   compared with aggregation occurring in the absence of
the respective antibodies. The mAb AMY-33, which
recognizes another epitope located within residues
1-28, affected the self-aggregation of β-peptide to a
lower extent of approximately 40%. The antibodies,
25   2H3 and 1C2, directed to the regions comprising
peptides 1-12 and 13-28, respectively, had a
considerably lower effect on *in vitro* amyloid
formation.
       On the basis of applicants findings
30   regarding these Examples and other antigen-antibody
systems studies (Solomon et al., 1989; Solomon and
Balas, 1991), the formation of the immunocomplexes
with selected, highly specific monoclonal antibodies,
should provide a general and convenient method to
35   prevent aggregation of the proteins without affecting
their biological properties.

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At least 15 different polypeptides are known to be capable of causing *in vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils.

5   Recent advances in antibody engineering technology, as well as in the development of suitable delivery systems (Haber, 1992; Pluckthun, 1992; Travis, 1993; Marasco et al., 1993) make it possible to develop functional small antibody fragments to

10   serve as therapeutic chaperones for the treatment of Alzheimer's disease as well as other human amyloidosis diseases by gene based therapies.

Application of the above findings for *in vivo* aggregation, can confer to single chain

15   antibodies (Pluckthun, 1992) or other engineered antibody fragments, a protective role in the renaturation of recombinant proteins.

Throughout this application various publications are referenced by citation or number.

20   Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to

25   which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of

30   limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the

35   invention may be practiced otherwise than as specifically described.

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CLAIMS

What is claimed is:

5   1.   A method of selecting anti-aggregation
molecules with chaperone-like activity that have
characteristics including binding to a native target
molecule epitope with a high binding constant and are
non-inhibitory to the biological activity of the
10   target molecule by
         denaturing a target molecule which
aggregates,
         mixing the target molecule with a
presumptive anti-aggregation molecule,
15   incubating the mixture under conditions
allowing for aggregation,
         selecting non-aggregated mixtures, and
         testing the nonaggregated target molecule
coupled to the anti-aggregation molecule for
20   bioactivity thereby selecting an anti-aggregation
molecule with chaperone-like activity.

         2.   The method of claim 1 wherein the anti-
aggregation molecule is a monoclonal antibody, a
25   genetically engineered antibody fragment or a peptide
which mimics the binding site for an antigen on the
antibody.

         3.   The method of claim 1 wherein the anti-
30   aggregation molecule is a single chain monoclonal
antibody.

         4.   The method of claim 1 wherein the target
molecule is β-amyloid.

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5.   The method of claim 1 wherein the target
molecule is a prion protein.

6.   A method of selecting anti-aggregation
molecules with chaperone-like activity that have
characteristics including binding to a target molecule
epitope with a high binding constant, reversing
aggregation effects and are non-inhibitory to the
biological activity of the target molecule by
          preparing an aggregated target molecule,
          mixing the target molecule with a
presumptive anti-aggregation molecule,
          selecting mixtures with non-aggregated
target molecules, and
          testing the target molecule coupled to the
anti-aggregation molecule for bioactivity thereby
identifying an anti-aggregation molecule with
chaperone-like activity.

7.   The method of claim 6 wherein the anti-
aggregation molecule is a monoclonal antibody, a
genetically engineered antibody fragment or a peptide
which mimics the binding site for an antigen on the
antibody.

8.   The method of claim 6 wherein the anti-
aggregation molecule is a single chain monoclonal
antibody.

9.   The method of claim 6 wherein the target
molecule is β-amyloid.

10.   The method of claim 6 wherein the
target molecule is a prion protein.

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11.   The method of treating a protein
aggregation disease including the steps of
          preparing at least one anti-aggregation
molecule that binds to an aggregating protein which is
5   the cause of a disease and which prevents aggregation
while allowing bioactivity;
          creating an expression vector comprising
nucleic acid including a sequence which encodes in
expressible form the anti-aggregation molecule that
10   binds to an aggregating protein and which prevents
aggregation while allowing bioactivity; and
          administering the expression vector.

12.   The method of claim 11 wherein the
15   anti-aggregation molecule is a monoclonal antibody, a
genetically engineered antibody fragment or a peptide
which mimics the binding site for an antigen.

13.   The method of claim 12 wherein the
20   monoclonal antibody that binds to an aggregating
protein and which prevents aggregation while allowing
bioactivity is a single chain monoclonal antibody.

14.   The method of claim 12 wherein the
25   monoclonal antibody that binds to an aggregating
protein and which prevents aggregation while allowing
bioactivity is a human anti-β-amyloid monoclonal
antibody.

30   15.   The method of claim 12 wherein the
monoclonal antibody that binds to an aggregating
protein and which prevents aggregation while allowing
bioactivity is an anti-prion protein monoclonal
antibody.

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16. The method of claim 11 wherein at least two anti-aggregation molecules are used.

17. The method of claim 11 wherein the
5   expression vector is a virus.

18. The method of claim 17 wherein the expression vector is a disable retro virus.

10   19. The method of claim 17 wherein the expression vector is a retroviral shuttle vector.

20. The method of claim 17 wherein the expression vector is vaccinia virus.
15

21. The method of claim 17 wherein the expression vector is an adenovirus.

22. The method of claim 17 wherein the
20 expression vector is a plasmid.

23. A pharmaceutical composition comprising an expression vector comprising nucleic acid including a sequence which encodes in expressible form an anti-
25 aggregation molecule that binds to an aggregating protein while allowing bioactivity and a pharmaceutically acceptable carrier.

24. The expression vector as set forth in
30 claim 23 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen.

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25.   The expression vector as set forth in claim 23 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

5   26.   The expression vector as set forth in claim 24 wherein the monoclonal antibody is a human anti-β-amyloid monoclonal antibody.

27.   An expression vector for use as a
10   therapeutic agent which comprises nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule that binds to an aggregating protein which is associated with a disease.
15

28.   The method of treating a protein aggregation disease including the steps of
        preparing at least one human monoclonal antibody that binds to an aggregating protein which is
20   the cause of a disease and allows bioactivity when bound, and
        administering the monoclonal antibody.

29.   The method of claim 28 wherein the
25   aggregating protein is β-amyloid.

30.   The method of claim 29 wherein the monoclonal antibody is an anti-β-amyloid and is selected from the group consisting of AMY-33 which
30   recognizes an epitope spanning amino acids 1-28 of β-amyloid and monoclonal antibodies 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β-amyloid peptide.

35

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31. A method of improving solubility and
yields in production of recombinant proteins by
        preparing an expression vector containing a
genetic sequence for an anti-aggregation molecule that
5   suppresses the kinetics of aggregate formation while
still encouraging formation of native protein
structure, and favors the desired folding reaction of
a recombinant protein,
        co-transforming a host cell with the
10 expression vector for an anti-aggregation molecule and
an expression vector for a recombinant protein thereby
increasing intracellular production of the fusion
proteins and preventing formation of inclusion bodies
and facilitating extracellular protein secretion.
15

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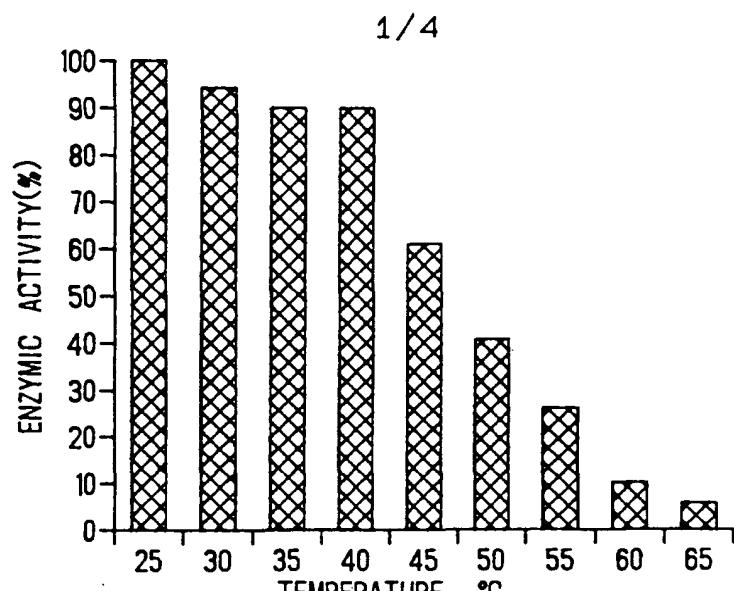
1/4

Fig-1

Fig-2

Fig-3

2/4
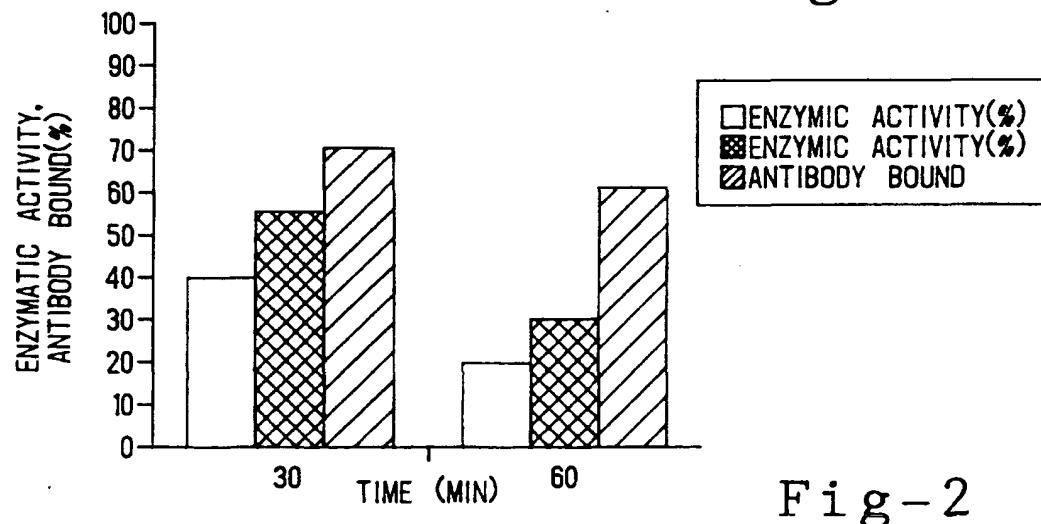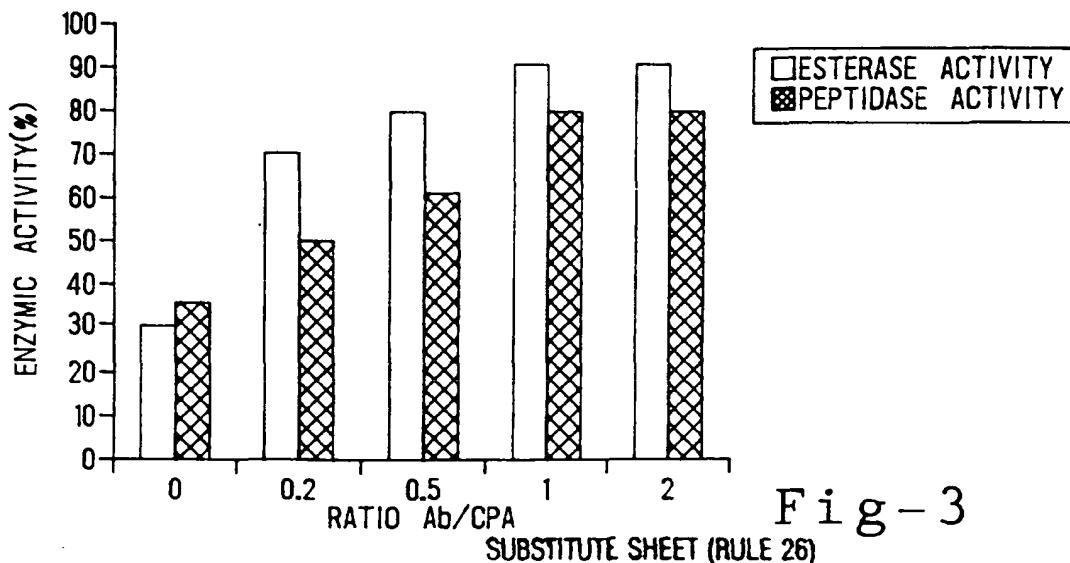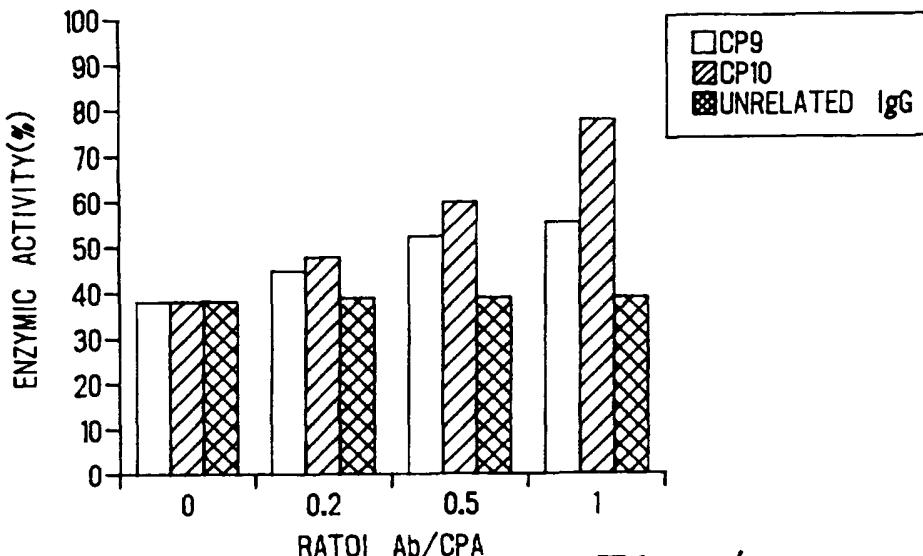
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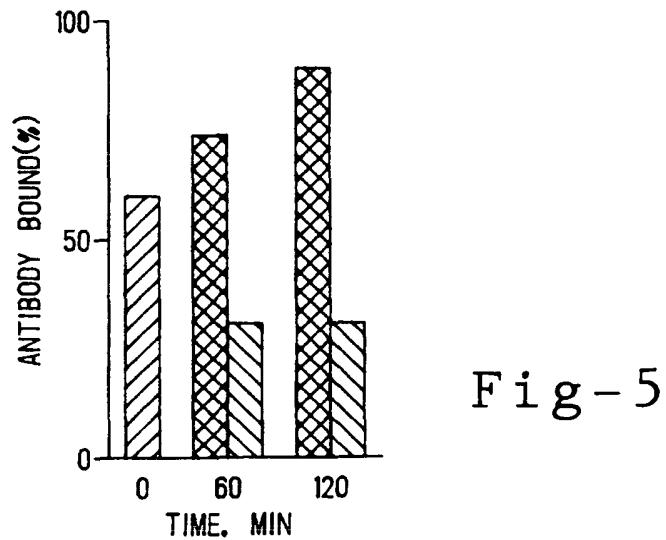
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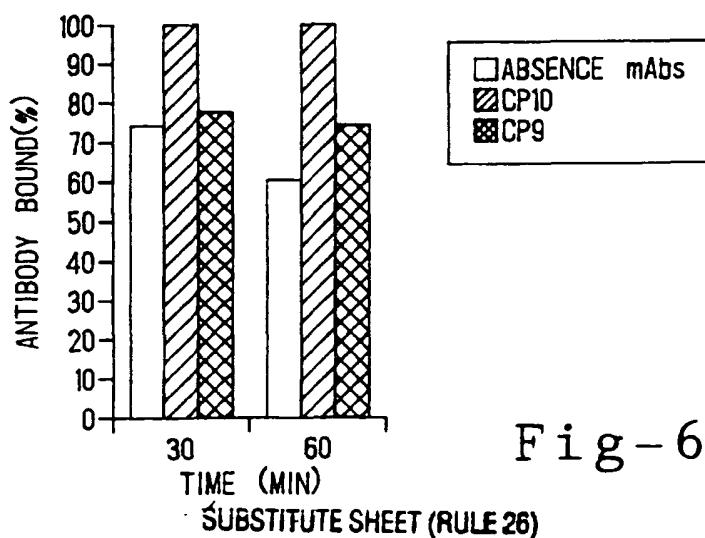
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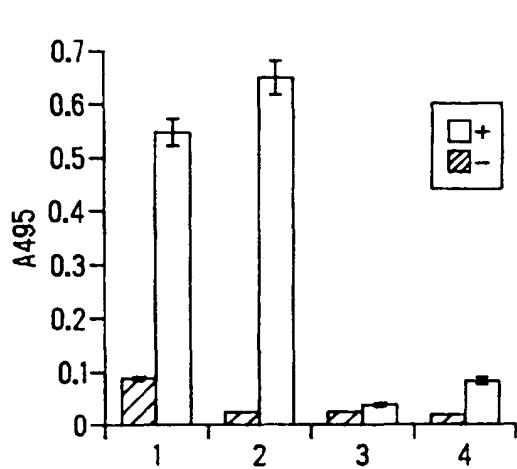
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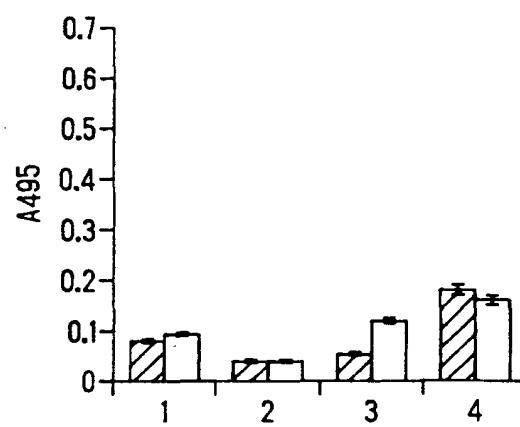
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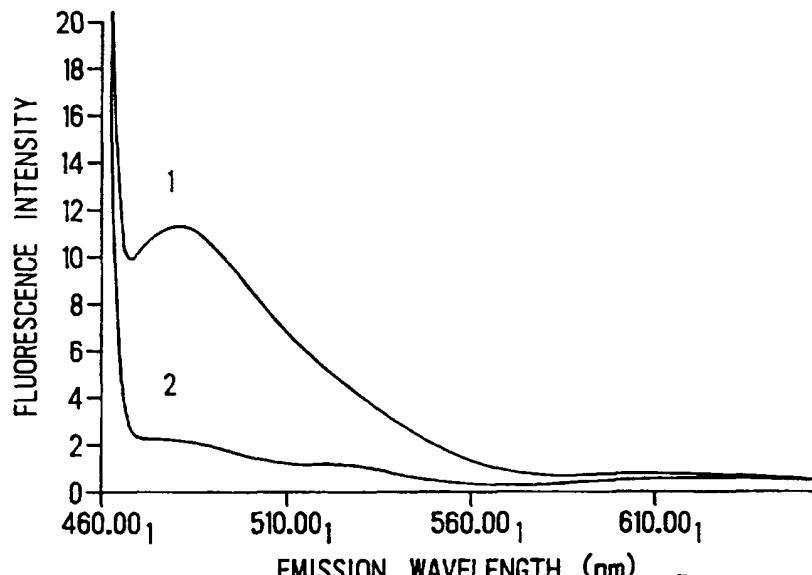
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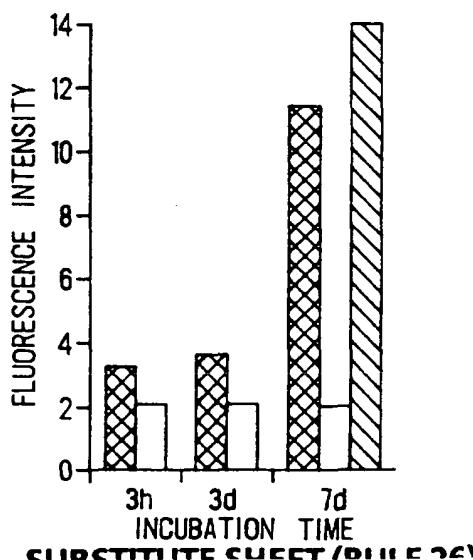
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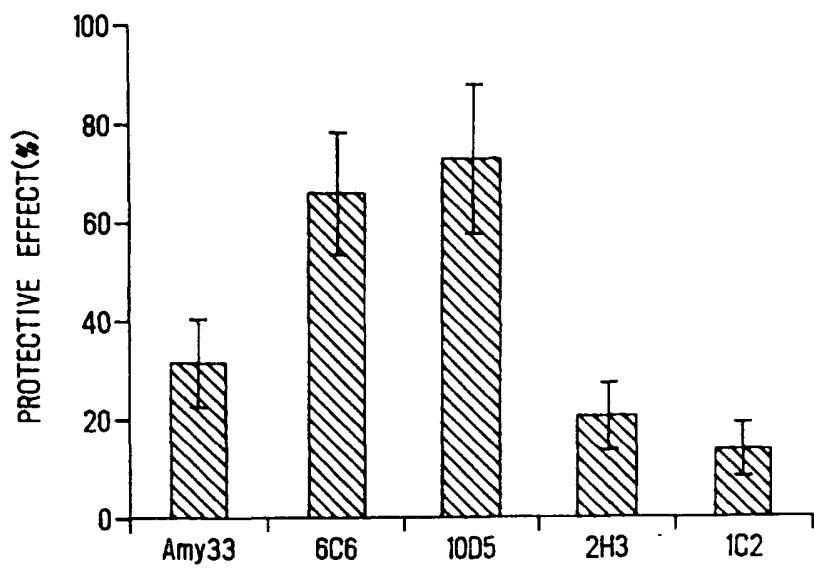
Fig-9

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53; A61K 38/00, 31/66; C12N 15/00; C12P 21/06
US CL :435/7.1, 69.1, 320.1; 514/12, 130

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 320.1; 514/12, 130

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, Volume 192, No. 2, issued 30 April 1993, Wisniewski et al, "Apolipoprotein E: Binding to Soluble Alzheimer's β-Amyloid", pages 359-365, especially pages 362-363.	1-30

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 APRIL 1996	23 APR 1996

Name and mailing address of the ISA/US	Authorized officer ~ /'. 𝒦𝓊𝓏𝒶 ᵖᵃ
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Form PCT/ISA/210 (second sheet)(July 1992)*

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
   ☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-10, drawn to a method of selecting anti-aggregation molecules.
Group II, claim(s) 11-21 and 27-29, drawn to a method of treating a protein aggregation disease.
Group III, claim(s) 22-26, drawn to a pharmaceutical composition and an expression vector.
Group IV, claim(s) 30, a method to improve solubility and yields in production of recombinant proteins.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: A special technical feature does not link the claims as the prior art reveals compounds which prevent protein aggregation (Wisniewski et al (1993) Biochem. Biophys. Res. Comm., 192, 359-365. There is no unity of invention absent a special technical feature which links the claims as provided in PCT Rule 13.2.